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High-risk human papillomavirus-positive lung cancer ***Molecular evidence for a pattern of pulmonary metastasis***

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ABSTRACT

Introduction

Infection with high-risk types of human papillomavirus (hrHPV) is associated with cervical, anogenital and oropharyngeal cancers. Since a causal contribution of hrHPV infection to lung cancer is still a matter of debate, a comprehensive study was performed to delineate hrHPV involvement in lung cancer using a Dutch study population.

Methods

Archival tissue specimens from 223 patients (145 males, 78 females, median age 65, range 27-87 years) who presented with cancer in the lungs were subjected to GP5+/6+-PCR and p16^{INK4A}-immunohistochemistry. The series included primary lung carcinomas of patients without a history of prior cancer (n=175), primary lung carcinomas of patients with an unrelated cancer in the past (n=36), and carcinomas with primary presentation in the lungs of which the origin (i.e., primary or metastasis) was equivocal at time of diagnosis (n=12). GP5+/6+-PCR/p16^{INK4A}-double-positive carcinomas were subjected to HPV genotyping, HPVE7 transcript analysis, loss of heterozygosity analysis and array comparative genomic hybridization.

Results

Whereas all primary lung carcinomas were hrHPV-negative (211/211, 100%), 3 hrHPV-positive equivocal carcinomas (3/12, 25%) were identified. These patients (1 male, 2 females) had a history of hrHPV-associated disease; one tonsillar and two cervical carcinomas. A clonal relationship between individual tumor pairs was supported by identical hrHPV genotype, pattern of p16^{INK4A} expression, HPVE7 mRNA expression, and genomic aberrations.

Conclusions

hrHPV presence in a tumor with primary presentation in the lungs signifies pulmonary metastasis from a primary, hrHPV-positive cancer elsewhere in the body. No support was found for an attribution of hrHPV infection to the development of primary lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide¹. Apart from etiological factors such as tobacco smoke exposure and (occupational) exposure to asbestos, radon, arsenic, and outdoor air pollution², viral agents have been proposed to be implicated in lung carcinogenesis³⁻⁵. Certain types of human papillomavirus (HPV) are firmly established as human carcinogens⁶. Infection with high-risk types of HPV (hrHPV) has been recognized as the main causative event for almost all cervical carcinomas⁷, a subset of other anogenital cancers, including vaginal, penile, vulvar, and anal cancers^{8,9}, and a proportion of oropharyngeal cancers¹⁰⁻¹². However, the association and therefore a potential etiological link between hrHPV infection and lung cancer remains controversial.

Many studies have reported on the prevalence of hrHPV in lung cancers with highly variable and conflicting data, that is, hrHPV prevalence figures ranged from 0-80% between studies and showed an average frequency of 20-25% worldwide¹³⁻¹⁶. Observed discrepancies might be explained by difference in geographical distribution of hrHPV prevalence and/or inter-laboratory variation regarding sample collection and sample handling methodologies. Next, differences regarding the method used for hrHPV detection are likely to contribute to the observed heterogeneity in prevalence worldwide. Previous studies evaluating a role of HPV infections in lung carcinogenesis addressed only part of the criteria that were postulated to prove a causal involvement of HPV in carcinogenesis^{17,18}. In particular, the sole demonstration of HPV DNA present in tumor specimens by means of PCR-based detection assays, as used in many studies, provides insufficient evidence for a causal relationship. As such, many aspects of the association between HPV infections and the development of lung cancer remain elusive.

A widely accepted "gold standard" for detection of a biologically relevant association between hrHPV infection and cancer is measurement of viral E6/E7 oncogene transcripts in tumor specimens. Expression of viral oncogenes E6 and E7 plays a crucial role in malignant transformation and maintenance of the transformed state by inactivating host tumor-suppressor genes p53 and pRB, respectively¹⁹⁻²¹. However, although measurement of viral oncogene expression can be reliably performed on snap-frozen samples, it is not readily feasible on routine formalin-fixed, paraffin-embedded (FFPE) material as mRNA isolates of FFPE sample origin are often of poor quality. Therefore, alternative methods have been proposed to assess HPV involvement using FFPE materials. A comparison of various (combinations of) methods has proposed p16^{INK4a}-immunohistochemistry (IHC) together with consensus primer hrHPV DNA PCR (i.e. GP5+/6+-PCR) to provide outcomes that are equivalent to transcript analysis on corresponding frozen samples²². This algorithm recently has been validated on a series of head and neck squamous cell carcinoma (HNSCC) FFPE specimens by showing 98% accuracy¹².

Using GP5+/6+-PCR together with p16^{INK4a}-IHC, the current study aimed to delineate hrHPV involvement in lung cancer using lung cancer specimens collected in a Dutch study population (n=223). The studied series included primary lung carcinomas of patients without a history of prior cancer (n=175), primary lung carcinomas of patients in whom another, unrelated primary

cancer had been diagnosed in the past (second primary lung cancer, n=36), and carcinomas with primary presentation in the lungs of which the origin (i.e., primary or metastasis) was equivocal at time of diagnosis (n=12). The GP5+/6+-PCR/p16^{INK4a}-IHC-double-positive tumor specimens were subjected to genotyping of GP5+/6+-PCR products, HPVE7 transcript analysis, loss of heterozygosity (LOH) and array comparative genomic hybridization (arrayCGH) analysis, to understand the clinical meaning of an active hrHPV infections in the lungs.

MATERIALS AND METHODS

Tissue specimens

Archival tumor tissue specimens from 223 patients (145 males, 78 females, median age 65, range 27-87 years) who presented with cancer in the lungs, were retrieved from the archive of the Department of Pathology, VU University medical center, Amsterdam, The Netherlands (Table 1). The series included, according to the clinical and pathology records of the patients, 211 primary lung carcinomas and 12 carcinomas with primary presentation in the lungs of which the origin was equivocal at time of diagnosis and may represent tumors with a primary or secondary manifestation in the lungs. The primary lung carcinomas were from patients without a history of prior cancer (n=175) or patients in whom another, unrelated primary cancer had been diagnosed in the past (second primary lung cancer, n=36). Information was obtained from the Dutch nationwide network and registry of histo- and cytopathology (PALGA, Utrecht, The Netherlands)²³. Collection, storage and use of archival tissue and patient data were performed in compliance with the “Code for Proper Secondary Use of Human Tissue in the Netherlands” (<http://www.fmwv.nl> and www.federa.org). This study followed the ethical guidelines of the Institutional Review Board (IRB) and was approved by the IRB.

Tissue processing and isolation of nucleic acids

Tissue sections were prepared according to the sandwich method in which the outer sections were stained by haematoxylin and eosin (H&E) for histological analysis, and in-between sections were used for p16^{INK4a} immunostaining (3µm) and nucleic acid isolation (10µm). Following deparaffinization of respective tissue sections, DNA was isolated using Proteinase K digestion (O/N at 52°C) followed by phenol-chloroform extraction, as described previously²⁴. Total RNA was extracted using TRIzol reagent (Life Technologies, Breda, The Netherlands) following manufacturer's instructions. Several precautions were taken to prevent false-positive test results. The microtome was cleaned between sectioning of separate specimens and a new microtome blade was installed for each specimen to avoid sample cross-contamination. Subsequent steps such as sample preparation and PCR amplification were performed in strictly separated rooms, and distilled water samples were included as negative PCR controls. None of the controls was positive for any of the HPV assays.

HPV DNA detection and genotyping

DNA isolates were subjected to GP5+/6+-PCR with an enzyme immunoassay (EIA) read-out using a cocktail of 14 high-risk HPV types (i.e., HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and 23 low-risk HPV types (i.e., HPV 6, 11, 26, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 70, 71, 72, 73, 81, 82/mm4, 82/is39, 83, 84, and cp6108) essentially as described before^{25,26}. Subsequent genotyping of EIA-positive samples was performed as described previously²⁶. Beta-globin PCR was performed on each DNA isolate as a quality control.

Detection of HPVE7 mRNA by Reverse transcriptase (RT)-PCR

Detection of type-specific HPVE7 mRNA was performed essentially as described before²⁷. Amplicon sizes were 109, 77, and 103bp, respectively, for HPV16, HPV18 and HPV33 (primers available upon request). RQ-1 DNase (Promega, Leiden, The Netherlands) treatment was applied to reduce the signal from contaminating genomic DNA. Besides DNase treatment, RT-reactions were also performed without reverse transcriptase added to confirm absence of residual DNA. RT-PCR was performed on each RNA isolate with primers specific for the housekeeping gene U1 small nuclear ribonucleoprotein specific A protein (snRNP U1A) as quality control²⁷. As a positive control, plasmid DNA of the respective HPV type was used in the PCR assay. All PCR products were visualized by gel-electrophoresis using 2% agarose gel.

Immunohistochemical staining of p16^{INK4A}

Immunohistochemistry was carried out using a mouse monoclonal antibody against p16^{INK4A} (clone 16P04 (JC2); Neomarkers, Fremont, USA) on a fully automated BOND-MAX (Leica Microsystems GmbH, Wetzlar, Germany) platform using the Bond Polymer Refine Detection system (Vision BioSystems, Newcastle, UK). The sections were pre-treated with Bond Epitope Retrieval Solution 2 (pH 9.0) (Vision BioSystems) for 20 minutes at 99°C. As a negative control, a mouse monoclonal antibody against Rat oxytocin-related neurophysin was used. All slides were examined by an expert pathologist (EBL) for the presence of p16^{INK4A} positive staining. Moderate to strong diffuse nuclear and/or cytoplasmic immunoreactivity in more than 70% of the carcinoma tissue was considered as p16^{INK4A} -positive, whereas tissue with only faintly diffuse or no to little (≤ 70%) reactivity was considered to be p16^{INK4A} -negative²⁸.

Loss of Heterozygosity (LOH) analysis

Twelve microsatellite markers were used to assess allelic loss on chromosome arms 3p, 9p, 11q and 17p: D3S1766, D3S1029, D3S1293, D9S171, D9S162, D9S157, D11S1883, D11S1369, D11S2002, CHRN1, TP53, and D17S1866. Multiplex PCR analysis was performed on 50 ng of DNA using sets of both labeled (FAM, HEX, NED) and unlabeled primers to amplify the microsatellite markers essentially as described before²⁹. The ABI 3130 Genetic Analyzer (Applied BioSystems BV, Nieuwerkerk a/d IJssel, The Netherlands) was used to separate the labeled PCR products and measure the signal intensities as described previously²⁹. Allelic loss was assigned if the signal intensity of one of the two alleles decreased with ≥50% compared with normal reference DNA isolated from non-neoplastic tissue of the same patient.

Array Comparative Genomic Hybridization

For arrayCGH purposes, genomic DNA was isolated from FFPE tumor tissue specimens using the column-based QIAamp DNA micro kit (Qiagen, Westburg, Leusden, The Netherlands) according to manufacturer’s protocol. The DNA isolation procedure was preceded by an overnight incubation (37°C) of the de-paraffinized tissue samples with sodium thiocyanate (1 M) to reduce the number of formalin induced DNA cross-links. Accordingly, normal mucosal DNA was isolated from FFPE biopsies obtained from 10 patients with non-malignant respiratory disease to be used as a pooled reference sample. Tumor and reference DNA (500ng) were labeled with either Cyanine 3-dUTP (Cy3) or Cyanine 5-dUTP (Cy5) nucleotide mixtures using the CGH labeling kit for oligo arrays (Enzo Life Sciences, Farmingdale, NY, USA). Sample hybridizations were performed using the Agilent SurePrint G3 human CGH array 4x180K platform containing 180,880 *in situ* synthesized oligonucleotides evenly distributed over the genome (spacing of ~17 kb) and 4,548 additional unique oligonucleotides, covering 238 of the Cancer Census genes (Agilent Technologies, Santa Clara, CA, USA). In brief, labeled samples were combined in a 110 µl mixture containing 39 µl of Cy3/Cy5 DNA mixture, 5 µg Cot-1 DNA (Invitrogen, Breda, The Netherlands), 10x Oligo aCGH Blocking agent and 2x Hi-RPM Hybridization buffer (both obtained from Agilent Technologies). Following incubation steps at 95°C for 3 min and 37°C for 30 min, the mixture was applied to the array-slide for hybridization (24 hr at 65°C). Array image acquisition and image analysis was done using an Agilent G2505B micro-array scanner and feature extraction software v10.7.3.1 (Agilent Technologies), respectively. Log₂ ratios (test/reference signal intensities) were calculated for each spot and normalized against the median of the ratios of all autosomes. Raw data are available through the Gene Expression Omnibus (GEO), accession number GSE40777.

ArrayCGH data and statistical analysis

ArrayCGH data analysis was performed using the statistical programming package R (v2.10.1). To overcome potential wave bias due to differences in GC-content of the different chromosomal regions, a smoothing algorithm was applied on our dataset³⁰. The CGHcall algorithm was used for recovering the segmental structure of normalized copy number profiles and defining the discrete copy number states of all chromosomal regions³¹. To distinguish somatic focal copy number aberrations from germline copy number variations (CNVs), all focal aberrations (< 3 Mb) that were identified using the called copy number data were compared with a database of known CNVs in the healthy population (database of genomic variants). To evaluate tumor clonality or independency between tumor pairs, the copy number profiles of two tumors were compared using two measures of similarity: the likelihood ratio (LR) using R package Clonality^{32,33}, and the Pearson’s correlation coefficient on segmented data using R.

RESULTS

Prevalence of high-risk HPV

All primary lung carcinomas were hrHPV-negative (211/211, 100%) (Table 1). Among the 12 carcinomas with equivocal origin at time of diagnosis (i.e., primary presentation of cancer in the lungs that may represent either second primary cancer or metastatic disease), 3 hrHPV-positive cancers (3/12, 25%) were identified. The hrHPV-positive specimens comprised: a p16^{INK4A}-immunopositive SCC with HPV16 in a female who had a history of cervical cancer (case 1); a p16^{INK4A}-immunopositive AdCa with HPV18 in a female who had a history of cervical cancer (case 2), and a p16^{INK4A}-immunopositive SCC with HPV33 in a male who had a history of tonsillar cancer (case 3; Table 2, Figure 1). In addition, one lung SCC (case 4) was found positive for low-risk HPV type 11 in a patient with a history of juvenile laryngeal papillomatosis from 9 years of age onwards. This tumor was p16^{INK4A}-immunonegative.

TABLE 1 Characteristics of study population						
Tissue specimens	Patient group	Histology	n	Gender (M / F)	Median age (years, range)	hrHPV-positive n (%)
Primary lung cancer	no history of cancer	AdCa	82	41 / 41	63 (35 - 84)	0 (0%)
		SCC	68	60 / 8	70 (47 - 87)	0 (0%)
		LCC	16	11 / 5	65 (47 - 74)	0 (0%)
		NOS	9	4 / 5	66 (52 - 70)	0 (0%)
	unrelated cancer in the past	AdCa	16	10 / 6	67 (48 - 80)	0 (0%)
		SCC	17	11 / 6	62 (27 - 77)	0 (0%)
		LCC	3	0 / 3	63 (61 - 74)	0 (0%)
		NOS	-	-	-	-
Total		211	137 / 74	65 (27 - 87)	0 (0%)	
Equivocal lung cancer	cancer in the past	AdCa	3	2 / 1	66 (58 - 70)	1 (12.5%)
		SCC	8	5 / 3	63 (39 - 74)	2 (66.7%)
		LCC	1	1 / 0	66 (66 – 66)	0 (0%)
		NOS	-	-	-	-
	Total		12	8 / 4	64 (39 - 74)	3 (25%)
AdCa, adenocarcinoma; F, female; hrHPV, high-risk human papillomavirus; LCC, large cell carcinoma; M, male; NOS, non-small cell lung carcinoma not otherwise specified; SCC, squamous cell carcinoma.						

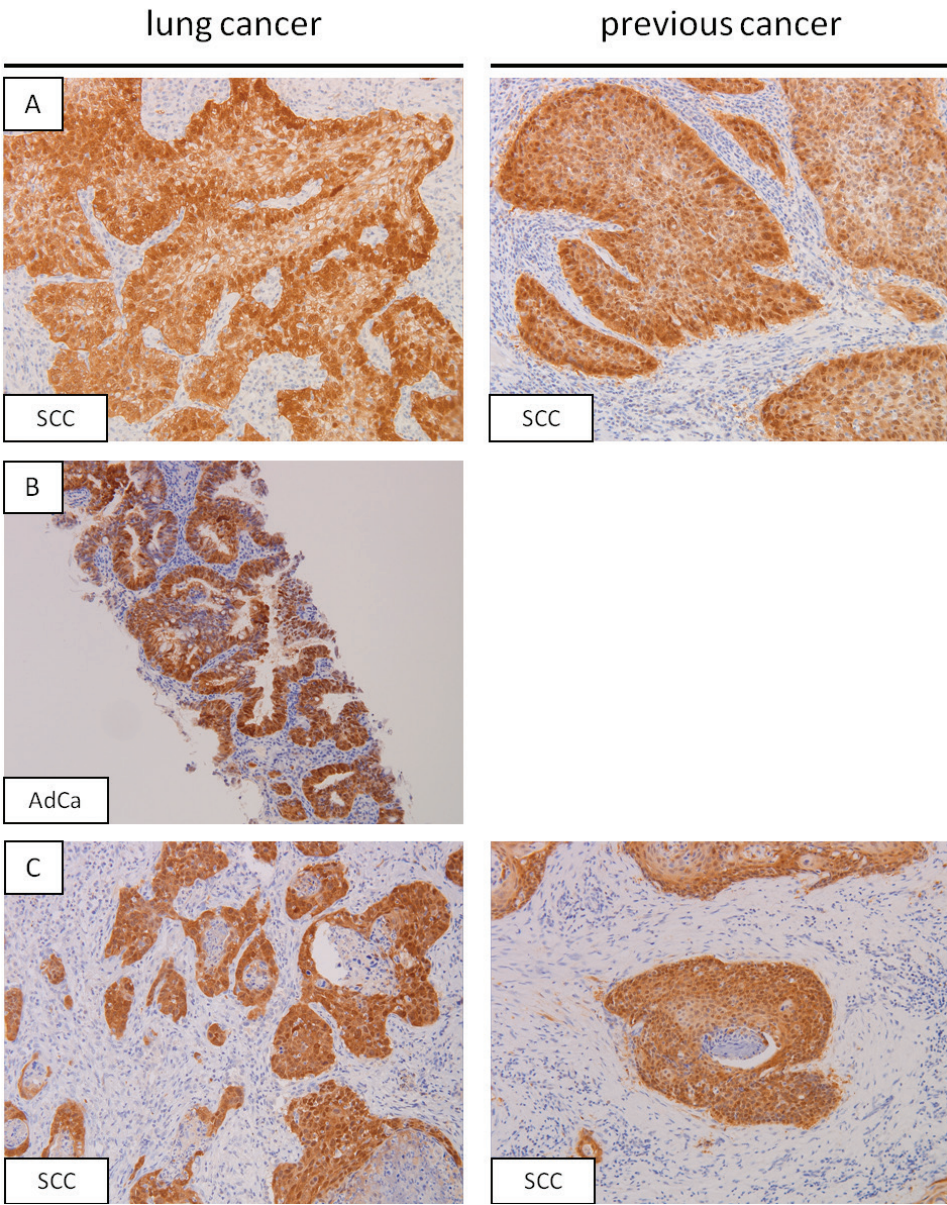


FIGURE 1 P16^{INK4A} expression patterns in tumor pairs. Immunohistochemical staining patterns for p16^{INK4A} are shown for (A) paired tumors of case 1, (B) lung cancer of case 2, and (C) paired tumors of case 3. Photographs were taken using a 100x magnification. AdCa, adenocarcinoma; SCC, squamous cell carcinoma.

Assessment of clonal relationship

Further molecular studies were performed to assess the likelihood of a clonal relationship between tumor pairs. For these analyses, we had available tissue material of the carcinomas that were previously diagnosed in cases 1 and 3 (i.e., cervical and tonsillar carcinoma, respectively). HPV status, HPV genotype and E7 viral oncogene expression showed concordant results with their respective lung carcinomas (Table 2). In addition, the pattern of p16^{INK4A} immunostaining and histomorphology were identical in tumor pairs (Figure 1). Microsatellite analysis at chromosomal regions 3p, 9p, 11q, and 17p demonstrated similar patterns of allelic (im)balance between tumor pairs in both cases. In case 1, 8 of the 12 markers were informative and revealed 6 concordant loci showing retention of heterozygosity (ROH), 1 concordant LOH at chromosome 9p (D9S157) and 1 locus (D3S1029) with acquired LOH in the lung tumor. In case 3, 10 of the 12 markers were informative and all showed concordant ROH between both tumors. Using arrayCGH analysis, DNA copy number profiles of respective tumor pairs were generated and vertically aligned according to genomic position as shown for both cases in Figure 2. Genomic profiles of tumor pairs demonstrated a highly similar appearance (Case 1; 40.6 vs. 31.6% aberrant genomic features; Case 3; 24.2 vs. 23.8% aberrant genomic features). The two tumor profiles of Case 1 revealed identical copy number gains at chromosomes 3q, 9p and identical copy number loss at 19p (Figure 2A). On top of this, identical focal aberrations were observed at chromosome arms 4p (gain of 4p16.3) and 9p (gain of 9p22.3-p22.1). Accordingly, tumor profiles of case 3 demonstrated identical chromosomal aberrations at chromosomes 3p and 20p supplemented with identical focal aberrations at chromosome arms 9p (loss of 9p23), and 14q (loss of 14q21.2) (Figure 2B). The degree of correlation between chromosomal segmentation patterns in both cases was marked (Pearson’s R = 0.68 and 0.39, respectively). The DNA copy number profiles from the analyzed tumor pairs were compared to a calculated reference histogram originating from selected pairs of independent tumors (according to ^{32;33}) and revealed a clonal relationship between paired tumors in both cases (i.e., Case 1: LR = 8.7, and Case 3: LR = 1.29).

TABLE 2 Overview of hrHPV-positive cancer cases

Patient ID (gender, age)	Lung carcinoma					Previous cancer			
	histo type	HPV type	HPVE7 mRNA	p16 ^{INK4A} expression	organ, histo type	TOD*	HPV type	HPVE7 mRNA	p16 ^{INK4A} expression
Case 1 (F, 52)	SCC	16	+	+	cervix, SCC	30	16	+	+
Case 2 (F, 57)	AdCa	18	+	+	cervix, AdCa	12	nd	nd	nd
Case 3 (M, 62)	SCC	33	+	+	tonsil, SCC	53	33	+	+

AdCa, adenocarcinoma; F, female; M, male; nd, not done (i.e., no tissue material available for analysis); SCC, squamous cell carcinoma; TOD, time of diagnosis; *, reported in months prior to the diagnosis of lung cancer.

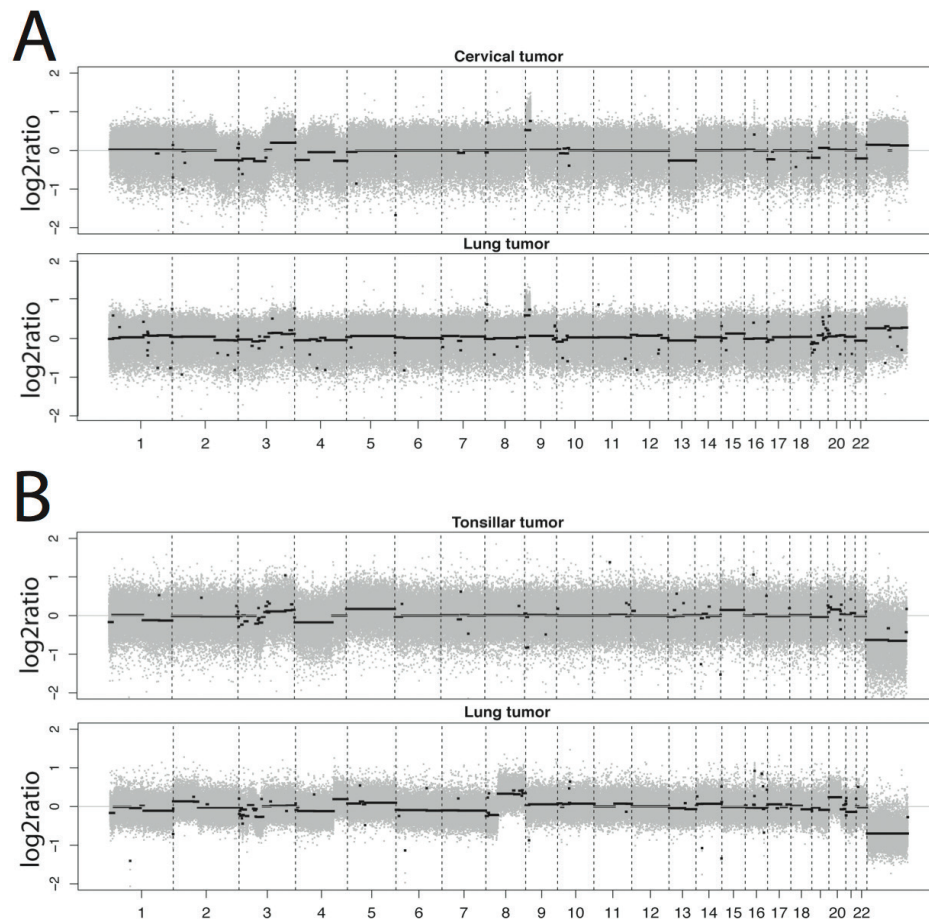


FIGURE 2 Genomic profiles support a common clonal origin in tumor pairs. DNA copy number aberrations for tumor pairs originating from (A) case 1 and (B) case 3 as detected by arrayCGH are shown. Genomic profiles were aligned according to chromosomal position (X-axis) and display log2 tumor to normal ratios (Y-axis). Both individual array measurements (180K in total, represented by gray dots) and respective segmented values (black lines) are indicated. Gain and loss are positive and negative log2ratio, respectively.

DISCUSSION

In the present study, no support was found for an attribution of an hrHPV infection to the development of primary lung cancer in a large series of primary lung cancer specimens from a Dutch population. We provided molecular evidence that hrHPV presence in fact signified pulmonary metastasis from a primary hrHPV-associated cancer source elsewhere in the body. Our findings imply that one should be aware of metastatic disease in case of an hrHPV-positive cancer with primary presentation in the lungs.

On the basis of our results, the prevalence of hrHPV in primary lung cancer is essentially 0%, which is in line with previous findings of Western population-based studies^{15;34;35}. The proof of pulmonary metastatic disease comprises an important clinical aspect. The lungs are the most frequent site of metastatic spreading in extra-pulmonary solid malignancy (i.e., 20-54% of all cases³⁶), and the differentiation between second primary cancer or metastatic disease often poses a problem in case of patients presenting with cancer in the lungs. Whether the presence of pulmonary metastasis of an hrHPV-associated cancer more generally accounts for the detection of hrHPV in lung cancer tissue, or whether geographical differences exist warrants further study, including patient history and molecular clonality analysis.

Whereas Asian studies have reported on an association between HPV, lung adenocarcinoma and *EGFR* mutation^{37;38}, the current study found no evidence for hrHPV in primary lung adenocarcinomas nor primary lung squamous cell carcinomas. In our series, 11 *EGFR* mutant primary lung adenocarcinomas (i.e., 7 cases with *EGFR* exon 19 deletion and 4 cases with *EGFR* exon 21 point mutation p.L858R; data not shown) were represented, which were all hrHPV-negative. The present study suggests no association between hrHPV and *EGFR* mutations in a Dutch population. The low viral loads found in the Asian studies^{37;38} makes it difficult to determine the etiological significance of their findings.

Our findings of pulmonary metastatic disease are in line with those of case studies reporting on the occurrence of hrHPV-positive pulmonary metastases of cervical cancer, HNSCC, and penile cancer³⁹⁻⁴¹. Based on the combination of morphology, HPV typing and tumor genetics, this study provides strong evidence for a clonal relationship between tumors, making a primary lung cancer highly unlikely. Our data underscore the value of p16^{INK4a} IHC together with GP5+/6+-PCR on FFPE specimens to measure a biologically relevant association between hrHPV infection and cancer^{12;22}. The low levels of allelic loss in the lung cancers further support the presence of a transcriptionally active hrHPV infection⁴². Our findings underline the valuable role of HPV genotyping, LOH analysis and arrayCGH analysis as diagnostic tools to discriminate primary from metastatic lung cancer^{39;40;43;44}.

In this study, one patient case was detected with low-risk HPV11 in the lung cancer tissue. The patient history showed laryngeal papillomatosis from early age onwards. HPV genotyping of the papillomatosis revealed HPV11 (data not shown) suggesting a direct association. Despite the fact that HPV11 is generally recognized as non-oncogenic, malignant transformation has been reported for individuals with juvenile-onset recurrent respiratory papillomatosis (RRP), with cancers particularly arising in the upper and lower respiratory tract⁴⁵⁻⁴⁷. Whether malignant transformation in recurrent respiratory papillomatosis is due to viral integration of HPV11⁴⁸ or is resulting from an alternative etiological factor, e.g. p53 mutation^{47;49} or hrHPV co-infection⁵⁰, remains inconclusive with only few reported studies. The latter, however, seems unlikely given the lack of p16^{INK4a} expression and non-detection of hrHPV DNA in our lung cancer case. A recent paper by Yuan *et al*⁵¹ reported on a mutant HPV11 genome with duplication of the promoter and oncogene regions in lung tumor tissue of a patient with RRP, and speculated that the duplication of the viral regions was associated with the clinical aggressiveness of the tumor in the patient.

Rearrangements of HPV11 genomes have originally been reported in a metastasis of lung cancer in a patient with juvenile-onset HPV 11-positive laryngotracheobronchial papillomatosis⁵². Yuan *et al*⁵¹ furthermore reported on vorinostat for the treatment of the HPV11-related lung cancer with stabilized tumor size with a durable effect at 15 months⁵¹. These findings hold promise for the treatment of HPV11-related lung cancers.

In summary, our results provide strong evidence for hrHPV-positive lung carcinoma being, in fact, a pulmonary metastasis from an hrHPV-associated primary carcinoma originating elsewhere in the body. Our data, together with the findings of previous studies^{15;34;35}, do not support a role for hrHPV infection attributable to primary lung carcinoma in Western populations.

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